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Metabolic Requirements for Microcycle Sporogenesis of *Bacillus megaterium*[1]

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Spores of *Bacillus megaterium* QM B1551 germinated, elongated, and resporulated (microcycle sporogenesis) in simple chemically defined media which permitted no cell division. The second-stage spores thus produced were heat-stable and required heat activation for germination. The orignal amount of spore deoxyribonucleic acid tripled before completion of the cycle. Acetate and a small amount of a tricarboxylic acid cycle intermediate were the minimal organic metabolic requirements for microcycle sporogenesis. During this cycle, germinated cells oxidized acetate only after a delay, whether or not glucose was initially present. Spores that were germinated in the absence of a carbon source first oxidized an endogenous substrate, and then developed the ability to oxidize acetate.

By diluting a complex medium shortly after spores had germinated, Vinter and Slepecky (14) demonstrated that the first cell formed on germination of a *Bacillus cereus* or of a *B. megaterium* spore was capable of resporulating without intervening cell division (microcycle sporogenesis). Microcycle sporogenesis may offer an optimally uncomplicated and synchronous system for the study of a morphogenetic cycle—the cycle from the spore stage through the vegetative state to the spore stage. The present work was done to discover the minimal nutritional requirements for this cycle in *B. megaterium*, to compare germination properties of spores produced in microcycle sporogenesis with those produced in complex media, and to determine whether the primary cell (the cell resulting from the germination of a spore) was initially repressed for acetate oxidation.

MATERIALS AND METHODS

Spores of *B. megaterium* QM B1551 were grown on a medium containing 0.5% Liver Fraction B (Wilson Laboratories, Chicago, Ill.) buffered at *p*H 6.5 with 10 mM potassium phosphate, and were harvested, washed, and lyophilized as previously described (9). Unless otherwise noted, spores for microcycle cultures were suspended at 30 mg/ml in water and heat-activated at 60 C for 10 min, when germinated with glucose, and 30 min, when germinated with iodide. Of the heated spore suspension, 0.1 ml was added to 2.9 ml of microcycle medium in 50-ml Erlenmeyer flasks to give a final spore concentration of 1.0 mg/ml. Cultures were incubated at 30 C on a shaker reciprocating 100 times/min with a 3-inch (7.6-cm) stroke.

The fraction of spores completing microcycle was determined by microscopic counts of sporangia in stained culture smears, or by a test for heat resistance (70 C for 10 min) involving the usual plating procedure. Cell counts were made in a Petroff-Hausser chamber.

Glucose was measured as total reducing sugar with dinitrosalicylate (12). Deoxyribonucleic acid (DNA) was extracted and measured by the diphenylamine method of Burton (3). Two perchloric acid extractions at 70 C removed the maximal amount of DNA from both spores and vegetative cells; salmon DNA was the standard. Dipicolinic acid (DPA) was estimated by the method of Janssen et al. (8). Oxygen uptake was determined by conventional Warburg techniques; 3 mg of spores in a total volume of 3 ml was used in each flask.

Cell extracts for enzyme assays were prepared by shaking 50 mg of spores or an equal number of primary cells together with 4.5 g of glass beads (No. 13 Ballotini, ca. 0.1 mm diameter) and 5 ml of 0.1 M tris(hydroxymethyl)-aminomethane (Tris) buffer (*p*H 7.2) for 40 sec in a Nossal cell disintegrator (McDonald Engineering Co., Cleveland, Ohio) at 4 C. This treatment ruptured more than 99% of the spores and cells. The homogenate was centrifuged at 10,000 × *g* for 20 min; the supernatant extract was used in the assays. Protein nitrogen was determined, by the nesslerization technique of Miller and Miller (10), as the trichloroacetic acid precipitable fraction of extracts.

Per cent germination was estimated by microscopic counts of cells staining with 0.5% methylene

[1] Some of the data in this paper were presented at the 66th Annual Meeting, American Society for Microbiology, Los Angeles, Calif. 1–5 May, 1966.

blue. The optical density of cultures was determined at 560 mμ.

Aconitase activity was measured spectrophotometrically (1).

Actinomycin D was generously provided by Merck Sharp and Dohme Research Laboratories, West Point, Pa.

RESULTS

Description of the cycle. A high percentage of microcycle sporogenesis occurred in a chemically defined medium containing 10 mM glucose, 3 mM NH_4Cl, 6 mM K_2HPO_4, and 1.5 mM K_2SO_4 (initial *p*H 7.6). Heat-activated spores (1 mg/ml) were 96% germinated after 15 min of incubation in this glucose-ammonia medium. With germination, the optical density of the culture and the heat resistance of the cells decreased (Fig. 1). The optical density increased as the primary cells emerged and enlarged. Another increase in optical density beginning at about 10 hr was correlated with the appearance of second-stage spores and heat resistance. The DPA content of the cells increased after the 9th hr when nonrefractile forespores became visible (Fig. 2). The DNA content of the cells began to increase between 1.0 and 1.5 hr, doubled by 4.0 hr, and tripled by the end of the microcycle (Fig. 2). A tripling of DNA during sporulation of *B. cereus* has also been described by Young and Fitz-James (15). There was no cell division in this medium; the total number of cells remained constant throughout the 18 to 20 hr of incubation. The cells that did not form second-stage spores neither divided nor lysed.

In the experiment represented by Fig. 1, about 75% of the originally germinating spores formed second-stage spores; in other experiments, as high as 99% sporulation was recorded. At 18 hr, the primary cell, now a mature sporangium, contained a second-stage spore and was still partially encased in the coat of the initial spore (Fig. 3). After 48 hr of incubation, the sporangia had lysed, leaving free second-stage spores.

Germination properties of second-stage spores. Washed, but not lyophilized, second-stage spores, free from sporangia, were tested for germination in potassium phosphate-buffered (50 mM, *p*H 7.6) glucose or L-alanine. Like the lyophilized initial spores, the second-stage spores required heat activation for rapid germination in glucose (Table 1). However, neither heated nor unheated second-stage spores responded to L-alanine.

Second-stage spores which were not heat-activated produced only about 25% as many colonies on Nutrient Agar (Difco) as did heat-activated second-stage spores (data not shown).

Heat-activated second-stage spores germinated

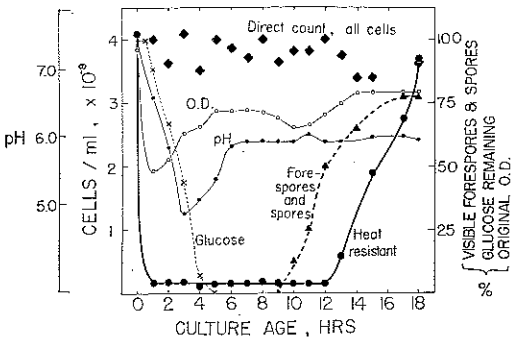

FIG. 1. *Events occurring during microcycle sporogenesis of Bacillus megaterium QM B1551 in glucose-ammonia medium.*

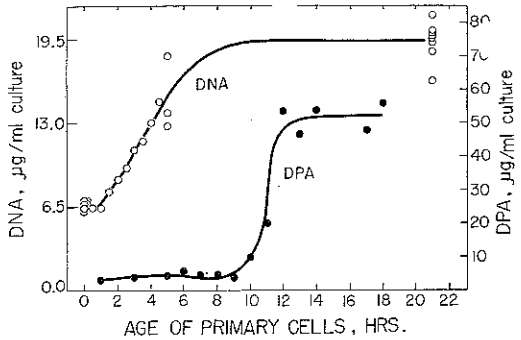

FIG. 2. *Concentrations of deoxyribonucleic acid (DNA) and of dipicolinic acid (DPA) extracted from primary cells of Bacillus megaterium QM B1551 undergoing microcycle sporogenesis in glucose-ammonia medium.*

and elongated, but were unable to complete microcycle sporogenesis in the glucose-ammonia medium which supported microcycle sporogenesis of initial spores.

Nutritional requirements for microcycle sporogenesis. (i) *Metals.* Initial spores contained a sufficient endogenous supply of divalent metals to support the formation of second-stage spores in the glucose-ammonia medium. Material released into the medium by the initial spores during early stages of germination was not required for subsequent sporulation. The sporulation cycle could be interrupted after 15 min of incubation (when 96% of the initial spores had germinated), and the germinated spores could be washed extensively in fresh glucose-ammonia medium, without altering the progress of the cycle.

(ii) *Phosphate.* No second-stage spores were formed in the absence of phosphate. In glucose-ammonia medium, buffered at *p*H 7.5 with 6 mM Tris or sodium cacodylate replacing phosphate,

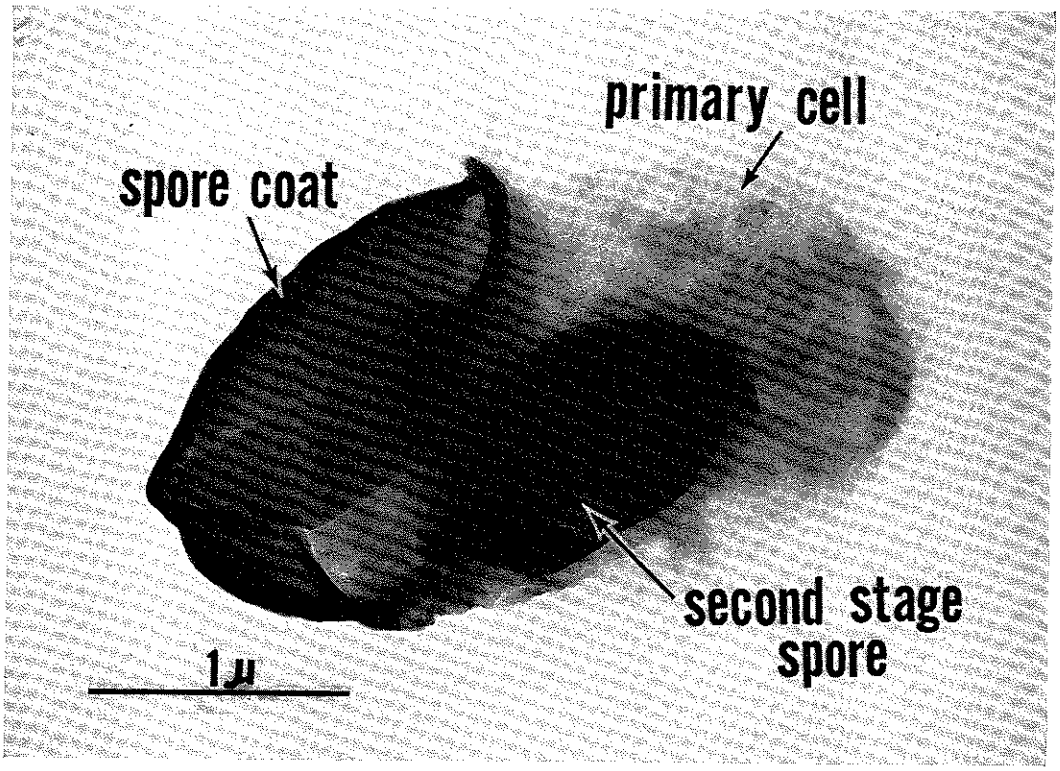

FIG. 3. *An 18-hr primary cell of Bacillus megaterium QM B1551 containing a second-stage spore. Shadowed with Cr at angle of 10°. The primary cell has emerged from the ruptured spore coat of the initial spore, and a smaller, mature, second-stage spore lies within it. Siemens Elmiskop 1A electron microscope, 80 kv, 50-μ objective aperture.*

glucose was oxidized very slowly, and at 24 hr the cells were swollen but not elongated [cf. Hyatt and Levinson (7)]. Normal formation of second-stage spores was supported by phosphate at concentrations from 5 to 50 mM in glucose-ammonia medium, or in glucose-ammonia medium containing 6 mM Tris.

(iii) *Sulfate.* No second-stage spores were formed in the absence of sulfate. Sulfate appeared to be necessary for the rapid oxidation of glucose [cf. Hyatt and Levinson (6)] and the rapid utilization of the acids produced; in sulfate-deficient glucose-ammonia medium, the *p*H of the culture fell and rose slowly, but visible forespores were not produced by 48 hr.

(iv) *Carbon and nitrogen sources.* The concentrations of the carbon and nitrogen sources in the glucose-ammonia medium described above (10 μmoles of glucose and 3 μmoles of NH_4Cl per mg of spores) allowed maximal second-stage sporulation. Increasing or decreasing the concentration of either compound separately impaired or delayed completion of the cycle (Table 2). Simultaneous increase in the concentration of both compounds resulted in cell division and in decreased sporulation of primary cells.

TABLE 1. *Germination of initial and second-stage spores of Bacillus megaterium QM B1551*[a]

Germinant	Germination			
	Initial spores		Second-stage spores	
	25 C	60 C	25 C	60 C
	%	%	%	%
Phosphate buffer.....	0	0	1	3
Glucose, 10 mM.....	4	84	2	63
L-Alanine, 100 mM....	11	57	4	2

[a] Aqueous suspensions of washed spores were treated for 10 min at 25 or 60 C before addition of germinant and incubation for 30 min at 30 C.

Glucose in the glucose-ammonia medium was oxidized during the first 4 to 5 hr of the sporulation cycle, the acids produced lowering the *p*H to

TABLE 2. *Effect of glucose and ammonia concentrations on the development of primary cells of Bacillus megaterium QM B1551*[a]

Concn		Forespores, nonrefractile	Refractile second-stage spores	Cell division
Glucose	NH_4Cl			
mM	mM	%	%	%
25	5	0	40	42
25	3	63	0	0
25	2	15	0	0
25	1	1	0	0
10	5	0	0	71
10[b]	3[b]	0	81	0
10	2	66	4	0
10	1	50	10	0
5	5	6	6	0
5	3	3	2	0
5	2	2	3	0
5	1	3	3	0

[a] Cultures were examined after 24 hr of incubation at 30 C. Data are percentages of all germinated cells.

[b] Represents concentrations used in the glucose-ammonia medium.

a minimum at 3 hr. The *p*H then rose as the acids were oxidized (Fig. 1).

Glucose, per se, was not required for microcycle sporogenesis. Ionically germinated (10 mM KI) initial spores formed second-stage spores in a medium containing, as carbon sources, 40 mM sodium acetate plus 1 mM concentrations of any of the following: glucose, ribose, citrate, isocitrate, glutamate, succinate, fumarate, or oxalacetate. Acetate alone did not support second-stage spore formation. Compounds which did not complement acetate for second-stage spore formation included ethyl alcohol, lactate, pyruvate, glycerol, dihydroxyacetone, α-ketoglutarate, malate, ribose-5-phosphate, 6-phosphogluconate, glucose-6-phosphate, L-methionine, L-cysteine, L-lysine, L-isoleucine, L-proline, DL-threonine, L-aspartate, and L-asparagine. An acetate-succinate medium containing 40 mM sodium acetate, 2 mM sodium succinate, 6 mM K_2HPO_4, and 10 mM KI (as germinant) supported 70 to 90% second-stage spore formation, and was used to study the development of the ability to oxidize acetate. In contrast to the situation with the glucose-ammonia medium, ammonia was not required in the acetate-succinate medium for the production of second-stage spores. No spores were produced in the presence of 2 mM succinate as sole carbon source.

Development of the ability to oxidize acetate. Acetate oxidation via the tricarboxylic acid cycle by primary cells appeared to be a prerequisite for microcycle sporogenesis, as it is for sporulation in other *Bacillus* species (5, 13). The competence to oxidize acetate apparently did not persist through the spore stage to the primary cell; primary cells developed this competence after germination.

Spores germinated ionically (10 mM KI) in acetate-succinate medium showed no acid-dependent oxygen uptake until 2 hr after germination (Fig. 4). The appearance of acid-dependent oxygen uptake was prevented by inhibitors of protein synthesis (chloramphenicol, 15 μg/ml; 8-azaguanine, 50 μg/ml; or actimomycin D, 50 μg/ml). It is inferred that enzymes necessary for acetate oxidation were synthesized after germination. One enzyme associated with acetate oxidation through the tricarboxylic acid cycle, aconitase, appeared only in the older primary cells (Table 3), a development which, in its relation to sporulation, is similar to that in *B. cereus* and *B. subtilis* (5, 13).

Primary cells needed no exogenous carbon source to develop competence to oxidize acids. Ionically germinated spores allowed to respire endogenously were able to oxidize, without delay, acids presented to them after 3 hr of incubation

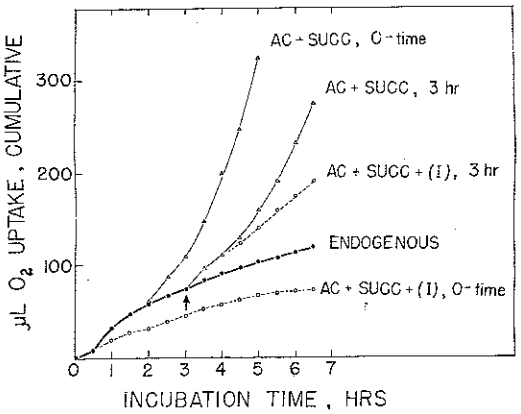

FIG. 4. *Oxygen uptake by primary cells under various conditions. Initial spores of Bacillus megaterium QM B1551 germinated with 10 mM KI, 6 mM K_2HPO_4, and 1.5 mM K_2SO_4 (endogenous). Acetate (AC), 40 mM, and succinate (SUCC), 2 mM, added with or without inhibitor (I) either at zero-time or after 3 hr of incubation at 30 C (arrow). Inhibitors (chloramphenicol, 15 μg/ml, or actinomycin D, 50 μg/ml) produced the same results whether added at the same time as, or 15 min prior to, the addition of the acids. Oxygen uptake on acetate (no succinate) was only slightly above endogenous (not shown). Sporulation occurred only in the cultures represented by the top two curves (AC + SUCC, 0-time; and AC + SUCC, 3 hr).*

(Fig. 4). By 3 hr, these endogenously respiring primary cells contained aconitase (Table 3).

Fluoroacetate, an inhibitor of acetate oxidation, decreased second-stage spore formation in glucose-ammonia medium (Table 4); this inhibition was relieved with citrate or succinate. Maximal fluoroacetate inhibition of sporulation occurred when the inhibitor was present before the *p*H had risen; fluoracetate added after 6 hr had less effect (Table 4).

The acid-oxidizing ability of spores incubated in the glucose-ammonia medium (as judged from the rise in *p*H) appeared as the glucose concentration was decreasing (Fig. 1). Adding extra glucose to the medium at any time before 9 hr of incubation resulted in renewed production of acid (Fig. 5) with complete suppression of second-stage sporulation. These additions of glucose did not promote cell division unless the medium was heavily buffered (50 mM PO_4^{-3}, *p*H 7.5). Addition of glucose after 9 hr inhibited further appearance of forespores.

TABLE 3. *Aconitase activity in extracts of spores and primary cells of Bacillus megaterium QM B1551*[a]

Age of cells	Aconitase activity per 10^8 cells	
	Glucose-ammonia medium	Carbon-free medium
hr	*unit*	*unit*
0	<0.02	<0.02
0.5	<0.02	—
3.0	2.59	1.67
4.0	2.88	—

[a] Prior to extraction, cells were incubated for the stated times in either glucose-ammonia medium or in carbon-free medium (10 mM KI, as germinant, substituted for glucose in glucose-ammonia medium). Cuvettes contained 10 μmoles of sodium DL-isocitrate, 100 μmoles of Tris buffer (*p*H 7.2), 0.05 ml of cell extract, in a volume of 3 ml. One unit represents a change in optical density at 240 mμ of 0.001 per min.

TABLE 4. *Inhibition of microcycle sporulation by fluoroacetate added at various times to spores and primary cells of Bacillus megaterium QM B1551 in glucose-ammonia medium*

Time of addition	Microcycle sporulation[a] (20 hr) with addition to culture of	
	Fluoroacetate (20 mM)	Fluoroacetate (20 mM) and succinate (40 mM)
hr	%	%
0	9	85
1	11	85
2	20	80
3	20	78
4	19	75
5	22	81
6	55	83

[a] With no additions to the culture, 75% of the cells completed microcycle sporulation.

Inhibition of sporulation by shift-up and shift-down. Increasing the available nutrients prevented microcycle sporogenesis. It has been seen that additional glucose suppressed sporulation, without promoting cell division. The addition of complex media, such as Nutrient Broth (Difco) or Arret-Kirshbaum (2) broth, at various times to primary cells in glucose-ammonia medium completely suppressed sporulation and promoted cell division as late as 6 hr, well after the oxidation of acids had begun. After 6 hr in glucose-ammonia medium, as cells became "committed" to sporulation, they became decreasingly capable of colony formation on solid complex media (Fig. 6). Primary cells were also less capable of forming colonies on the complex solid media for a period between 2 and 6 hr (Fig. 6), but they could still divide. For instance, 4-hr cells examined in liquid culture divided once after addition of complex medium and then either ceased division or lysed. Vinter and Slepecky (14) have reported a similar sensitivity to shift-down, rather than to shift-up, in primary cells of *B. cereus*.

In the glucose-ammonia medium a temporary shift-down was achieved (for the period from

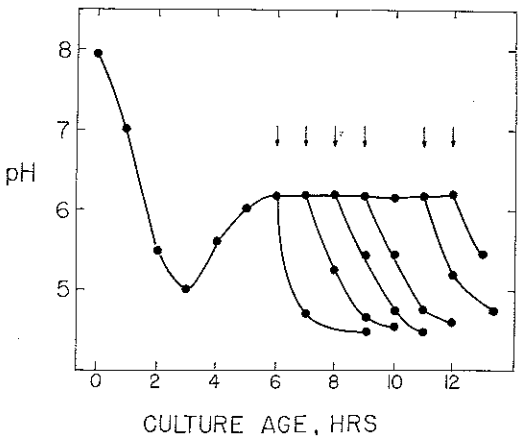

FIG. 5. *Changes in pH of microcycle cultures of Bacillus megaterium QM B1551 in glucose-ammonia medium supplemented at intervals with 10 μmoles of glucose per ml of culture. The upper curve represents pH in the unsupplemented culture; arrows indicate addition of glucose to separate cultures.*

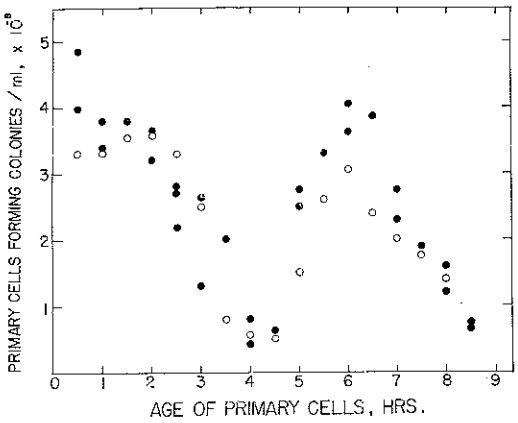

FIG. 6. *Colony-forming ability of Bacillus megaterium QM B1551 primary cells, taken from glucose-ammonia medium at intervals and plated on Nutrient Agar* (●) *or Arret-Kirshbaum agar* (○).

0.25 to 1.25 hr) either by chilling the culture to 2 C, or by incubation with 2 mM rather than 10 mM glucose. When the temperature of the chilled culture was restored to 30 C, the cells elongated and formed division septa, but did not sporulate. Similarly, when the normal glucose concentration was returned to the glucose-deprived culture, the cells elongated but failed to sporulate.

DISCUSSION

The endogenous reserves of *B. megaterium* QM B1551 were oxidized upon germination (Fig. 4), but did not support either cell division or sporulation. The minimal organic requirements for microcycle sporogenesis, acetate and a tricarboxylic acid cycle intermediate, were inadequate for continued growth. In this respect, primary cells resembled the late vegetative stage granulated cells of *B. cereus* (11), which sporulated, but did not multiply, in a medium containing acetate, citrate, and glutamate. The granulated cells, however, contained a functional tricarboxylic acid cycle; the newly germinated primary cells described here apparently did not.

A functioning tricarboxylic acid cycle was probably a prerequisite for microcycle sporulation. Fluoroacetate inhibited sporulation only when added before the acids were oxidized, and inhibition was relieved with tricarboxylic acid cycle components. Although extracts of spores of *B. megaterium* contain appreciable amounts of isocitric dehydrogenase, fumarase, and malic dehydrogenase (Holmes and Levinson, *unpublished data*), at least one of the enzymes involved in the tricarboxylic acid cycle, aconitase, was absent during germination, but was present during acid oxidation. Since ionically germinated cells oxidized acetate rapidly only in the presence of small amounts of tricarboxylic acid cycle intermediates, we infer that glucose, in the glucose-ammonia recycling medium, may serve to supply the newly germinated cell with priming amounts of 4-carbon compounds for the operation of the tricarboxylic acid cycle.

Acetate oxidation and sporulation are apparently repressed in newly germinated primary cells as well as in log-phase vegetative cells. In the case of spores germinated in the absence of a carbon source, the repression and derepression of acetate oxidation were apparently under the control of endogenous metabolism. Whether endogenous repression played a role when glucose was present has not been determined.

The inhibitory effects of chilling and of temporary glucose deprivation on microcycle sporulation may be related by a common influence on the initial rate of cell development; both treatments probably lowered the rate of glucose turnover. Yet glucose turnover was not a requirement for sporulation, as cells which germinated in the absence of a carbon source later sporulated successfully in acetate and succinate. The presence of glucose during early stages of recycle may have committed the cell to a phase of development which must be completed before sporulation can begin, and which, if temporarily interrupted by a premature lowering of the glucose concentration, proceeds at a decreased rate.

Grelet (4) has shown that *B. megaterium* is derepressed for sporulation when starved for any one of several compounds, including glucose, phosphate, sulfate, or nitrate, and has concluded that "sporogenesis is the response of a genetically apt bacterium to an increase of the generation time" (cf. R.S. Hanson, Bacteriol. Proc., p. 96, 1966). Under microcycle conditions, the generation time is extended indefinitely; in the absence of sufficient nutrient for cell division, nuclear replication proceeds and the primary cell sporulates.

ACKNOWLEDGMENT

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